PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 1352/4381	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EE 03/00005	International filing date *(day/month/year)* 16.09.2003	Priority date *(day/month/year)* 17.09.2002
International Patent Classification (IPC) or both national classification and IPC C07K16/46		
Applicant INBIO OÜ, et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06.04.2004	07.01.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Mossier, B Telephone No. +49 89 2399-8706

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EE 03/00005

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-4, 7, 10	as originally filed
5, 6, 8, 9	as amended (together with any statement) under Art. 19 PCT

Claims, Numbers

1-18	as amended (together with any statement) under Art. 19 PCT

Drawings, Sheets

1/4-4/4	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☒ furnished subsequently to this Authority in written form.

   ☒ furnished subsequently to this Authority in computer readable form.

   ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application,

   ☒ claims Nos. 18

   because:

   ☒ the said international application, or the said claims Nos. 18 relate to the following subject matter which does not require an international preliminary examination (specify):

   see separate sheet

   ☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the Standard.

   ☐ the computer readable form has not been furnished or does not comply with the Standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-18
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-18
Industrial applicability (IA)	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations

see separate sheet

The present application relates to the use of therapeutic antibodies entering into the cell. The claimed antibodies are conjugated to a cell penetrating transport peptide such as Transportan, TP10 or to peptide sequence comprising 9 Arginines. Putative anti-GLI1 and anti-GLI3 conjugated antibodies are disclosed, however no experimental data showing the therapeutic use of said antibodies are provided.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 18 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).
For the assessment of present claim 18 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claim. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item V

Reasoned statement under Article 35(2) PCT with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

V.1 The following documents were taken into account:

D1: ZHAO Y ET AL: "Chemical engineering of cell penetrating antibodies" JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 254, no. 1-2, 1 August 2001 (2001-08-01), pages 137-145, XP004245448 ISSN: 0022-1759

D2: WO 98/52614 A (ROTHBARD JONATHAN B ;UNIV LELAND STANFORD JUNIOR (US); WENDER PAUL) 26 November 1998 (1998-11-26)

D3: WO 01/12655 A (KAROLINSKA INNOVATIONS AB ;TOFTGAARD RUNE (SE)) 22 February 2001 (2001-02-22)

D4: GREEN J ET AL: "Basal cell carcinoma development is associated with induction of the expression of the transcription factor Gli-1" BRITISH

JOURNAL OF DERMATOLOGY, vol. 139, no. 5, November 1998 (1998-11), pages 911-915, XP002265619 ISSN: 0007-0963

D5: LINDGREN M M ET AL: "Cell-penetrating peptides" TRENDS IN PHARMACOLOGICAL SCIENCES, ELSEVIER TRENDS JOURNAL, CAMBRIDGE, GB, vol. 21, no. 3, March 2000 (2000-03), pages 99-103, XP004202572 ISSN: 0165-6147

V.2. According to the opinion of the International Examining Authority (IEA) the inventive concept underlying the present application is already disclosed in D1. D1, which can be considered as closest prior art, discloses a novel method to allow antibodies to penetrate the cellular membrans of living cells without affecting cell viability. For these purposes a membrane translocating sequence (MTS) has been covalently attached to antibodies using site-specific crosslinking technique. D1 further refers to a potential therapeutic use of said chemically engineered antibodies (Abstract; page 137, column 2, paragraph 1 - page 138, column 1, paragraph 1).
Hence, D1 immediately renders the subject-matter referred to in claim 5, 6, 16 and 18 obvious. Therefore, said claims appear to lack inventive step (Article 33(3) PCT).

V.3 With regard to D1, the problem to be solved by the present application can be regarded as the provision of further cell penetrating antibodies.
Since GLI proteins, anti-GLI1 antibodies as well as their specific use in medicine is already disclosed in prior art (see D3: page 11, line 30 - 12, line 23; claims 11 - 13), it would be obvious and with reasonable expectation of success for the person skilled in the art to combine the teaching of D1 with the subject-matter referred to in D3. Hence, claims 1, 3, 4, 8, 13 and 17 are considered to lack inventive step in accordance with Article 33(3) PCT.
Same applies for the subject-matter relating to anti-GLI-3 antibodies (see D4).

V.4 Moreover, since cell-penetrating peptides such as Transportan, TP10 and 9 Arginine are already disclosed in the prior art (see D2 and D5) it comes within the scope of the customary practice followed by persons skilled in the art to use said cell-penetrating peptides as membran translocating sequences, especially as the advantages thus achieved can readily be foreseen. Hence, claims 2 and 14 are not considered to involve an inventive step according to Article 33(3) PCT.

V.5 The subject-matter of claims 7, 9 - 12 and 15 that refer to different variable sequences of the antibody, respectively different "types" antibodies is also not

considered to be inventive, since said features are merely several straightforward possibilities from which the skilled person would select, in accordance with circumstances, without the exercise of inventive skill, in order to solve the problem posed. Consequently, the subject-matter of claim 7, 9 - 12 and 15 also appears to lack inventive step (Article 33(3) PCT).

Certain Observations on the International Application

*The following remarks on **Clarity and Sufficiency of Disclosure** (Article 6 and 5 PCT) are made:*

1) *The present application does not disclose therapeutic use, respectively a medical application of the claimed antibodies. Hence the subject-matter referred to in claims 5 - 15 and 18 is purely speculative and said claims are not considered to fulfill the requirements of Article 5 and 6 PCT.*

Moreover, the present invention is also directed to a method for the treatment of a disease or health disorder in humans or animals. Such method comprises the administration of a pharmaceutically acceptable dose of the invented molecule to humans or animals.

The above-mentioned pharmaceutical composition can be administered orally, intravenously or intraperitoneally. The preferred route of administration is intravenous.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the Transportan 10 (TP10) HPLC chromatogram (figure 1A) and the MALDI-TOF spectrum (figure 1B).

Figure 2 shows the mouse antiGLI IgG-~~Transportan~~ TP10 conjugate (figure 2A), the mouse FITC-conjugated anti-IgG (figure 2B), the mouse anti-GLI1(IgG)-Transportan TP10 conjugate (figure 2C), the mouse FITC-conjugated anti-IgG (figure 2D).

Figure 3A shows the production and purification of the cell penetrating recombinant protein. The figure shows the image of Coomassie brilliant blue-stained SDS-polyacryl-amide gel. Lane 1: molecular weight marker. Lane 2: uninduced *E.coli* cell lysate; Lanes 3 and 4: cell lysate, where the expression of the construct has been induced by IPTG. Lanes 5-8: protein fractions 1-4 eluted from glutathione-agarose.

Figure 3B shows the internalisation of the recombinant protein into human 293 cells. The cells were incubated with recombinant proteins and fluorescent anti-GST antibodies (upper image) detected their internalisation into the cells. The image below depicts the phase-contrast image of the same field.

DETAILED DESCRIPTION OF THE INVENTION

We have produced monoclonal antibodies against GLI1 and GLI3 proteins. We have conducted preliminary studies and demonstrated that antibodies coupled with cell penetrating transport peptides, are able to effectively penetrate the cell membranes and that

the coupling of such peptides to antibodies does not reduce the ability of the antibodies to recognise specific antigens.

Example 1. Obtaining and characterisation of polyclonal GLI1 antibodies

Polyclonal antibodies recognising the GLI1 protein were obtained by immunisation of rabbits by using the GLI1(1-407) antigen expressed in bacteria by using standard methods. The antibodies obtained were characterised by using the Western blot analysis, Electrophoretic mobility shift assay (EMSA) and immunohistochemical methods.

Example 2. Obtaining and characterisation of polyclonal GLI3 antibodies

Polyclonal antibodies recognising the GLI3 protein were obtained by immunisation of rabbits by using the GLI3(150-250) antigen expressed in bacteria and by using standard methods. The antibodies obtained were characterised by using the Western blot analysis, Electrophoretic mobility shift assay (EMSA) and immunohistochemical methods.

Example 3. Conjugation of peptides entering into the cell to polyclonal GLI1 antibodies

The CPP (Transportan 10 (TP10), the shorter analogue of transportan, was synthesised in 0.1 mmol scale on the Applied Biosystem Model 430A peptide synthesizer using the dicyclohexyl carbodimid/hydroxy-benzo-triazole (DCC/HOBT) activation. Peptides were cleaved from the resin according to the TFMSA cleavage protocol. Resulting peptide was further purified on C_{18} reversed-phase HPLC column that yielded >99% pure product. The molecular mass of each synthetic peptide was determined by MALDI-TOF mass spectrometry and the obtained result was compared with the calculated molecular mass. Transportan 10 (TP10), the shorter analogue of transportan, was conjugated to polyclonal antibodies. Figure 1 shows the conjugation of cell penetrating peptides to antibodies, which was carried out as follows:

1) CPP was derivatized into maleimid. SMCC solution (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, Mw 334; 1/5 molar ratio) was added to 200 ml of peptide solution in phosphate buffer (ph 7.5; 10 mg peptide/ml). The mixture described above was incubated for 1-2 hours at room temperature. The SMCC residue was removed by using the HPCL reverse-phase C_{18} column.

2) In order to deprotect the thiol groups on the antibody, TCEP (tris(2-carboxyethyl)phosphine hydrochloride; Mw 287) in 1/5 molar ratio, was added to the

Example 5. Obtaining and characterisation of anti GLI3 monoclonal antibodies

Monoclonal antibodies recognising the GLI3 protein were obtained by immunisation of rabbits with GLI3(150-250) antigen. The protein was expressed in bacteria and according to standard methods (Antibodies: A Laboratory Manual; Ed. Harlow, David Lane; Cold Spring Harbor Laboratory Press, ISBN: 0879693142). The resulting antibodies were characterised by Western blot analysis, electromobility shift assay (EMSA) and immunohistochemical methods.

Example 6. Developing a technology for obtaining recombinant cell penetrating proteins

For obtaining a recombinant cell penetrating protein we created expression vector encoding for GST-GLI3(150-250) fusion protein. We used PCR based approach to add the sequences encoding for cell penetrating peptides ~~Transportan~~ TP10 and ~~9Arg (9Arginine)~~ 9Arginine (9Arg) into previously mentioned vector. These expression constructs were sequenced. Expression of these constructs showed that despite repeated efforts, it was not possible to express a recombinant fusion protein that encoded GST-GLI3(150-250)-Transportan TP10 sequence described above in *E. Coli* system. We succeeded, though, in expressing and purifying a recombinant protein that encoded the recombinant GST-GLI3(150-250)-9Arg cell penetrating peptide (figure 3A).

As we have demonstrated on figure 3B, the obtained recombinant protein entered the cultured mammal cells.

Example 7. Obtaining and characterisation of anti GLI recombinant proteins entering into the cell

The recombinant antibodies were obtained by inserting the sequence encoding for the 9Arg peptide or Transportan or ~~Transportan~~ TP10 into the gene encoding the clones of antibodies described above. The obtained recombinant antibodies were purified using affinity chromatography and antibody titre was determined. We demonstrated that these antibodies were binding specifically to the GLI1 protein. These recombinant antibodies also entered into the eukaryotic cells in culture.

In order to obtain the scFv with ability to penetrate into the cell we made a construct encoding for single chain antibody, or scFv, containing the two variable domains of an

antibody molecule (the VL and the VH domain) linked via flexible peptide linker that also contained the sequence of CPP. The RNAs from the anti GLI1 and 3 monoclonal antibodies were reverse transcribed and this first cDNA strand was used as a template for variable regions amplification using degenerated primers:

T A/C A C C A T G G G A T G G A G A/C T G G A
ATTATCACTGGGTCACTTGAC
TGACAGGCTGGGCTGGCAGGA
A G C/T C T C C C C C/G A/T G G/A G/C C/T T
C T T G C A C A G A/T A A T A C A
GAGCTCGTGATGACCCAGTCTCCA
T T C C A G C T T G G T C/G C C A/G C C A/T
AACACTCATTCCTGTTGAAGC

PCR products of the appropriate size (320-350 bp) were purified and sequenced. Oligonucleotide primer encoding for Transportan or ~~Transportan~~ TP10 and linker $(Gly4Ser)_3$ was used to construct a VL-TP-Linker-VH sequence by three-step overlap extension PCR. The process was repeated for scFvFc construction with relevant VLCL and VHCH1 PCR products. The final PCR products corresponding scFv and scFvFc (both with CPP and linker encoding) sequence were cloned into eukaryotic expression vector (pcDNA3, pCEP) and sequenced. Eucaryotic cells (Cos-7) were be transfected with scFv or scFvFc constructs and according to the manufacturer's instructions for generation of stable cell lines. Recombinant proteins were purified from supernatant using Ni+ columns.

References:

Dahmane, N., Lee, J., Robins, P., Heller, P., and Ruiz i Altaba, A. (1997). *Nature* **389**, 876-81.

Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Unden, A. B., Sandstedt, B., Toftgard, R., and Zaphiropoulos, P. G. (1999). *Nat Cell Biol* **1**, 312-9.

Lecerf, J. M., Shirley, T. L., Zhu, Q., Kazantsev, A., Amersdorfer, P., Housman, D. E., Messer, A., and Huston, J. S. (2001). *Proc Natl Acad Sci U S A* **98**, 4764-9.

Claims

1. Molecule comprising at least (a) a scFv-part of an antibody recognising an intracellular GLI-protein, and (b) a cell penetrating transport peptide, wherein the two parts are chemically conjugated to each other.

2. Molecule according to claim 1, wherein the cell penetrating transport peptide comprises at least a part of Transportan, TP10 or 9Arginine.

3. Molecule according to claim 1, wherein the GLI-protein is chosen from GLI1 or GLI3.

4. Molecule according to claim 1-3, for medical use.

5. Use of a molecule comprising at least a part of an antibody recognising an intracellular antigen and a cell penetrating transport peptide for the preparation of a medicament for treatment of a cancer state.

6. Use according to claim 5, wherein the cancer state is chosen from skin cancer.

7. Use according to claim 5 or 6, wherein the antibody is monoclonal or polyclonal.

8. Use according to any one of claims 5 to 7, wherein the antibody has been obtained against the GLI1 or GLI3 protein.

9. Use according to claim 5, wherein the molecule comprises a variable sequence of a genetically modified antibody, to which the cell penetrating transport peptide has been conjugated or has been included in another manner in the sequence thereof.

10. Use according to claim 9, wherein the variable sequence is derived from the human genome.

11. Use according to claim 10, wherein the variable sequence that is derived from the

human genome is obtained by immunisation with an antigen of a transgenic animal having the humanised immune system.

12. Use according to claim 10, wherein the variable sequence that is derived from the human genome is obtained by screening a human antibody expression library with the intracellular antigen.

13. Use according to any one of claims 10 to 12, wherein a GLI1 or GLI3 sequence is used as the antigen.

14. Use according to any one of claims 5-13, wherein the cell penetrating transport peptide comprises Transportan, TP10, or 9Arginine.

15. Use according to any one of claims 5-14, wherein any intracellular therapeutic target is used as the antigen.

16. Pharmaceutical composition comprising at least one molecule as defined in claims 1 to 4 or as defined in claims 5 to 15, in association with at least one pharmaceutically acceptable carrier or additive.

17. Method for obtaining a recombinant antibody comprising at least (a) a scFv-part of an antibody recognising an intracellular GLI-protein, and (b) a cell penetrating transport peptide, wherein the method comprises the steps of expressing the recombinant antibody and purifying the obtained antibody.

18. Method for treatment of a disease or health disorder in humans or animals, comprising administrating a pharmaceutically acceptable dose of the molecule as defined in claims 1-4 or as defined in claims 5-15 to humans or animals.